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ORIGINAL ARTICLE

POU2AF1, an amplification target at 11q23, promotes growth of multiple myeloma cells by directly regulating expression of a B-cell maturation factor, TNFRSF17

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Multiple myeloma (MM), a progressive hematological neoplasm, is thought to result from multiple genetic events affecting the terminal plasma cell. However, genetic aberrations related to MM are seldom reported. Using our in-house array-based comparative genomic hybridization system to locate candidate target genes with following their expression analysis, we identified *POU2AF1* at 11q23.1 as a probable amplification target in MM cell lines. POU2AF1 is a B-cell-specific transcriptional co-activator, which interacts with octamer-binding transcription factors Oct-1 and Oct-2, and augments their function. Downregulation of POU2AF1 expression by specific small-interfering RNA (siRNA) inhibited MM cell growth, whereas ectopic expression of POU2AF1 promoted growth of MM cells. Among putative transcriptional targets for POU2AF1, B-cell maturation factor, TNFRSF17, enhanced its transcription by POU2AF1, and POU2AF1 directly bound to an octamer site within the 5′ region of *TNFRSF17*. Expression level of *TNFRSF17* was closely correlated with that of *POU2AF1* in cell lines and primary samples of MM, and decreasing TNFRSF17 expression by means of *TNFRSF17* siRNA inhibited MM cell growth. Taken together, our results suggest that POU2AF1, when activated by amplification or other mechanisms, may contribute to progression of MM by accelerating growth of MM cells through direct transactivation of one of its target genes, *TNFRSF17*.

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Introduction

Multiple myeloma (MM) is a malignancy characterized by clonal expansion of plasma cells, although clinical features, responses to treatment and survival times vary widely among MM patients. Despite advances in systemic and supportive therapies, MM remains an incurable disease. Cytogenetic and molecular studies have provided evidence of marked heterogeneity of chromosomal aberrations in MM cells (Seidl *et al*., 2003). In particular, translocations involving the immunoglobulin heavy chain (*IGH*) locus at 14q32 and different chromosomal partners occur in approximately 60% of MM cases (Fonseca *et al*., 2004). The most often recurrent *IGH* translocations in MM patients include t(11;14)(q13;q32), t(4;14)(p16.3;q32), t(6;14)(p21;q32), t(14;16)(q32;q23) and t(14;20)(q32;q11), which, respectively, deregulate the *CCND1*, *FGFR3* and *MMSET*/*WHSC1*, *CCND3*, *MAF* and *MAFB* genes. However, these translocations are not sufficient for malignant progression of MM; therefore, an accumulation of additional genetic alterations affecting tumor-related elements, such as proto-oncogenes and tumor-suppressor genes, appears to be necessary for the emergence of a fully malignant phenotype. Unraveling the nature and sequence of these events may have a critical impact on identification of the molecular mechanisms leading to distinct disease entities, potentially to allow definition of prognostic risk groups and development of targeted therapies of this disease.

Changes in genomic copy numbers can influence expression or activity of MM-associated genes. Comparative genomic hybridization (CGH) experiments using arrayed genomic DNAs (array-CGH), such as those derived from bacterial artificial chromosome (BAC) and/or P1-artificial chromosome (PAC) libraries, is currently one of the most powerful tools for detecting and simultaneously localizing losses or gains of genetic material throughout the genome (Inazawa *et al*., 2004). Combined with the human genome database, array-CGH enables high-throughput quantitative measurement of DNA copy numbers at high resolution, allowing rapid discovery of novel tumor-suppressor genes and proto-oncogenes.

In the course of a program to screen a panel of MM cell lines for copy-number aberrations in a genome-wide manner using an in-house BAC/PAC array, we identified amplification at 11q23. Frequent copy-number aberrations at 11q23 and/or 11q25 in primary MM have been reported previously (Cremer *et al*., 2005a, b), but the presumptive target genes have never been identified; this prompted us to perform a detailed search for specific amplification target(s) involved in the pathogenesis of MM. Within the 3-Mb smallest region of overlap (SRO) at 11q23, we successfully identified POU2AF1, also known as OBF-1/OCA-B/BOB.1, which is a B-cell-specific transcriptional co-activator that interacts with the octamer-binding transcription factors Oct-1 and Oct-2, and augments their function (Luo *et al*., 1992; Luo and Roeder, 1995).

Here we provide evidence to show that *POU2AF1* is a likely target for amplification in MM, although its amplification/overexpression was a relatively infrequent event in this disease. Increases and decreases in POU2AF1 expression respectively promoted and inhibited growth of MM cells in our experiments. Moreover, expression of TNFRSF17, also known as B-cell maturation antigen, was directly transactivated by POU2AF1, and down-regulation of TNFRSF17 inhibited growth of MM cells that overexpressed POU2AF1. The expression levels of *POU2AF1* and *TNFRSF17* were tightly correlated in MM cell lines as well as in primary specimens. These results suggested that overexpressed POU2AF1 through amplification or other mechanisms promotes growth of MM cells, at least in part, by way of its effect on the amount of TNFRSF17 in the plasma cell, thereby functioning as an oncogene during myelomagenesis by activating this transcriptional target.

Results

Array-CGH analysis of MM cell lines

We assessed copy-number aberrations in each of the 28 MM cell lines by array-CGH analysis using our in-house BAC/PAC-based array, MCG Cancer Array-800 (Inazawa *et al*., 2004). Figure 1a shows the frequencies of copy-number gains and losses across the entire genomes of all 28 lines. Gains of DNA that occurred in more than 50% of clones were most often at 1q, 7q and 8q; the most frequent losses were mainly at 1p, 13q, 14q, 17p and 22q. Representative genes in these clones are *MYC* (8q, 55%), *RB1* (13q, 81%) and *TP53* (17p, 51%), all of which are often aberrant in MM cell lines and primary MM cells. The most frequent losses we observed were in the 14q32 region, where the *immunoglobulin heavy constant gamma 1* gene (*IGHG1*) is located (88.9%, Figure 1a; Supplementary Table S1). Translocation in MM is mainly mediated by errors in two different gene-rearrangement mechanisms; it can result in the excision of DNA in the interval between a particular *V* gene and the corresponding *C* gene (Kabat, 1972). The prevalence of *IgH* translocations is about 85% in primary plasma cell leukemias and more than 90% in MM cell lines (Fonseca *et al*., 2002; Kuehl and Bergsagel, 2002). Therefore, we speculated that the deletions of *IGHG1* we observed were probably due to translocations, because of the similarity in frequencies.

We chose to focus our attention on remarkable patterns of chromosomal abnormalities such as high-level amplifications ($\log 2$ ratio >2.0) and homozygous deletions ($\log 2$ ratio <-2.0), which are likely to be landmarks of oncogenes and tumor-suppressor genes, respectively (Supplementary Table S1). Homozygous deletions had occurred in 11 of the 28 cell lines; *MTAP* and *CDKN2A/p16* at 9p21.3 were both homozygously deleted in cell line KMS-5. Two regions of homozygous deletion on 11q22 and 11q24 were also detected, and representative genes in those regions were *YAP1* and *FLI1*, respectively. The former deletion occurred in four MM cell lines (KMS-20, KMS-28BM, KMS-28PE and MOLP-6), whereas the other occurred only in one, KMS-28PE. Both deletions have also been reported recently in cell lines and primary tumor of MM by array-CGH using oligonucleotide array platform (Carrasco *et al*., 2006), although some of the cell lines overlapped with those in our panel. High-level amplifications were detected in 5 of the 28 MM lines, and 16 genes (clones) were represented (Supplementary Table S1). High-level amplifications at 11q23, in clones containing *POU2AF1* and *PPP2R1B*, were detected in two cell lines (AMO1 and MOLP-2, Figure 1b; Supplementary Table S1). Since amplification is one of the mechanisms that activate pathogenetically and clinically relevant genes, and overexpressed/activated genes can be good targets for developing novel therapeutic approaches, we chose to analyse the 11q23 amplification further.

Definition of the 11q23 amplicon in MM cell lines by fluorescence in situ *hybridization*

To generate a defined map of the 11q23 amplicon, we performed fluorescence *in situ* hybridization (FISH) analyses in two of our MM cell lines (AMO1 and MOLP-2) that had exhibited remarkable copy-number gains in this region, using 15 BACs spanning the amplified region and BAC RP11-100N3 locating at 11q11 as a control (Figures 2a and b). In AMO1 cells, four BACs (RP11-25I9, 262A12, 686G14 and 792P2) produced the highest number of signals as homogenously staining regions (HSRs) on marker chromosomes, whereas three BACs (RP11-262A12, 686G14 and 792P2) showed HSR patterns in MOLP-2 cells (Figures 2a and b; Supplementary Figure S1). Therefore, the SRO could be defined between BAC RP11-25I9 and 708L7 as the critical region. According to information gained from human genome databases (http://www.ncbi.nlm.nih.gov/ and http://genome.ucsc.edu/), the maximal size of this region of interest is approximately 3 Mb.

Expression of candidate target genes within the 11q23 amplicon in MM cell lines

Genes activated by copy-number increases and those involved in the progression of tumors are likely to be

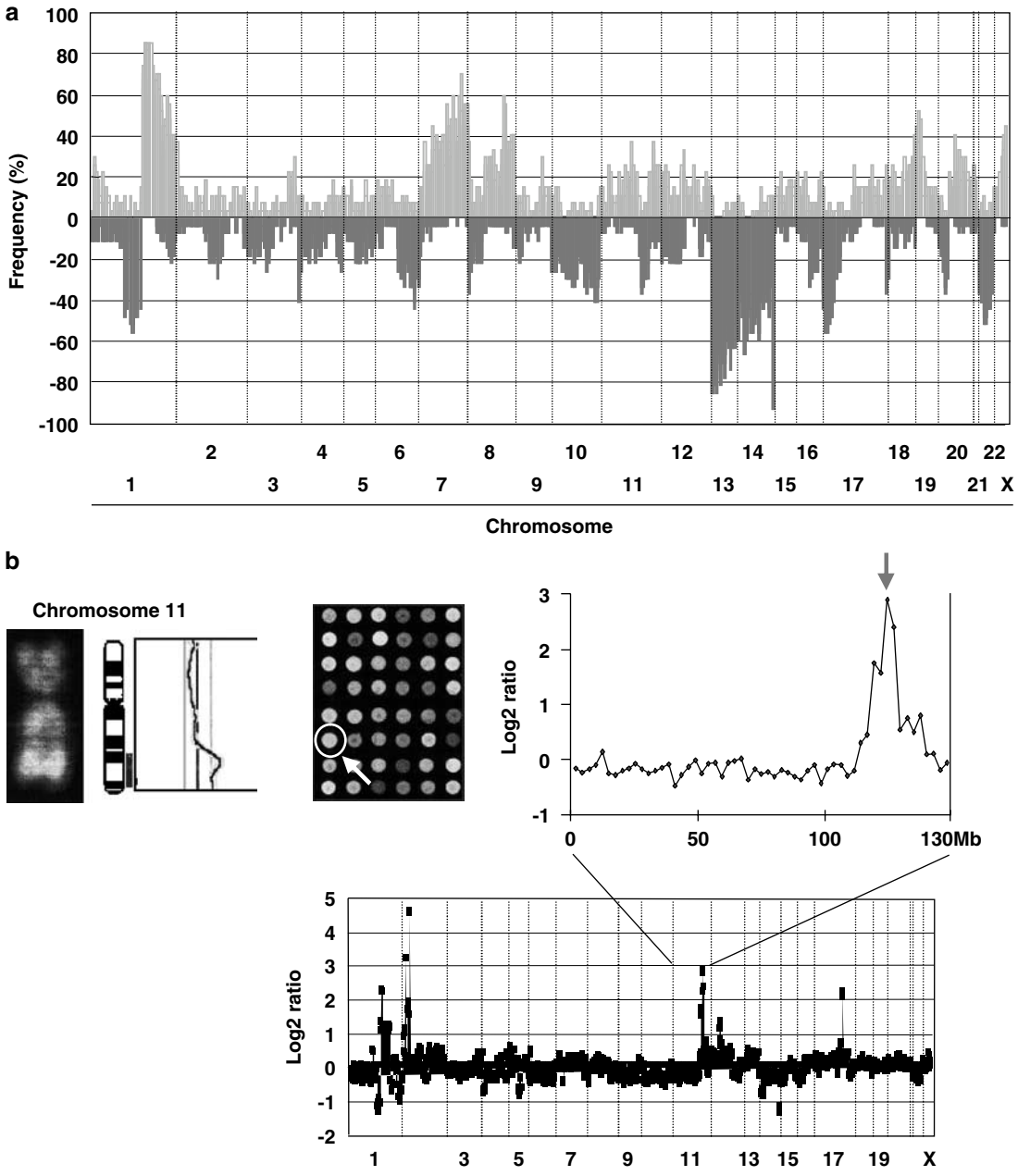

Figure 1 *POU2AF1* amplification detected in multiple myeloma (MM) cells by array-comparative genomic hybridization (CGH) analysis. (**a**) Genome-wide frequencies of copy-number gains (above 0, green) and losses (below 0, red) in 28 MM cell lines. Clones are ordered from chromosomes 1–22, X and Y, and within each chromosome on the basis of the UCSC mapping position (http://genome.ucsc.edu/ (version May 2004)). (**b**) Representative images of conventional CGH analysis (left) and array-CGH analysis of chromosome 11 in AMO1 (MM) cells along with a copy-number profile of the chromosome (upper right) and the whole genome (lower right) of this cell line. A conventional CGH analysis reported previously (Inoue *et al*., 2004) showed broad amplification at 11q, whereas we detected a specifically increased copy numbers of *POU2AF1* at 11q23 as a clear green signal (log2 ratio = 2.8) on the MCG Cancer Array-800. Arrows indicate the spot containing the *POU2AF1* gene.

located in the SROs of amplicons. To determine whether genes amplified at 11q23 were overexpressed in association with amplification, we assessed the expression status of each transcript located within 3-Mb SRO in MM cell lines as described previously (Inoue *et al*., 2004). On the basis of our amplicon map, nine known genes were selected from the genome databases (Figure 2b and Table 1). The expression level of each transcript determined by reverse transcription–PCR (RT–PCR), normalized with *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), in AMO1 and MOLP-2 cell lines was divided by the average expression level of each transcript found in two MM cell lines that had exhibited normal copy numbers at 11q23 (KMM1 and KM-5, data not shown), and recorded as a 'fold increase' (Table 1). Fold increases larger than 2.0 were considered

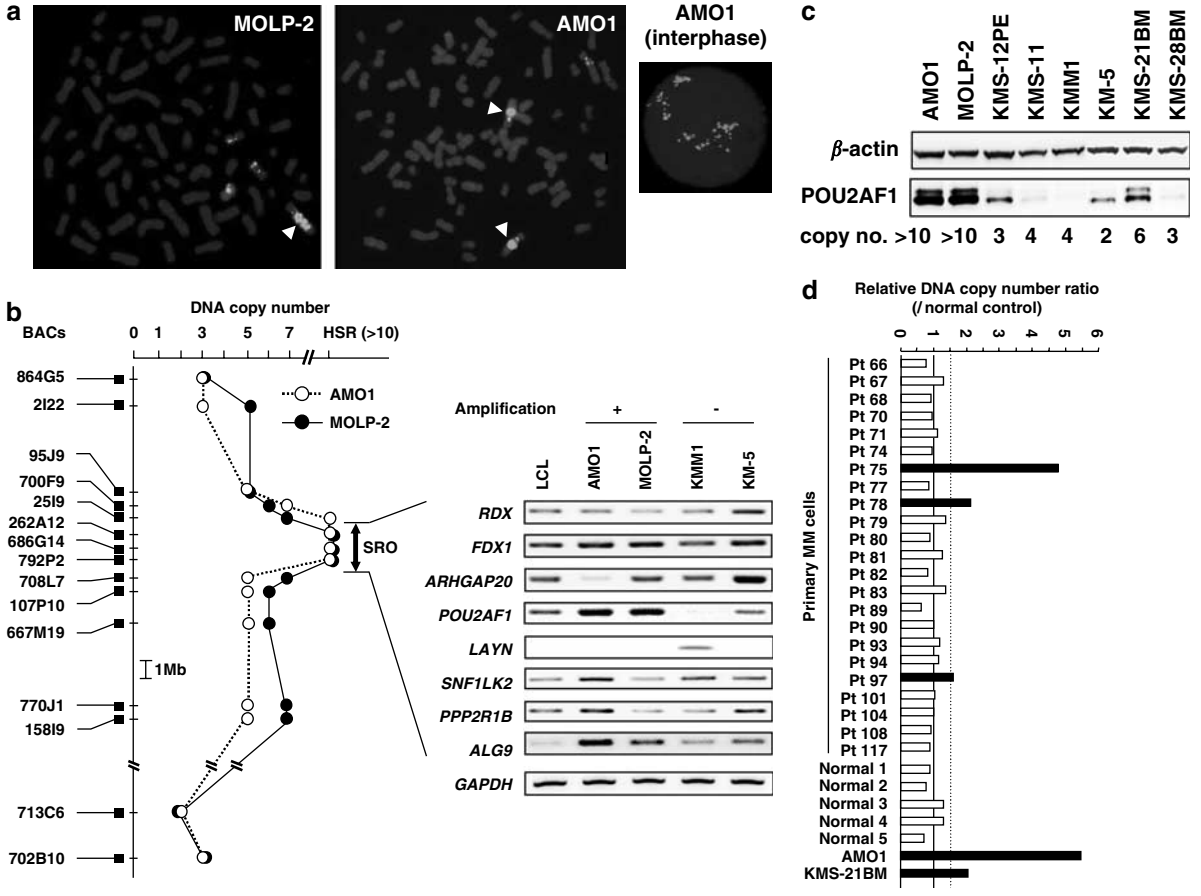

Figure 2 Amplification and subsequent overexpression of POU2AF1 in multiple myeloma (MM) cell lines. (**a**) Representative image of fluorescence *in situ* hybridization (FISH) analyses on metaphase chromosomes from MOLP-2 (left) and AMO1 (middle) cells and the interphase chromosome from AMO1 cells (right). Bacterial artificial chromosome (BAC) RP11-792P2 (green signals), which contains the *POU2AF1* gene, shows copy number increases in both cell lines, with a homogenously staining region (HSR) pattern on marker chromosomes (green signals, arrowheads). Control BAC RP11-100N3 close to centromere (11q11) showed red signals in MOLP-2 cells and in AMO1 cells. (**b**) Summarized results of DNA copy-number analysis by FISH in two different MM cell lines. (Left) Map of 11q23 covering the amplified region. The location of each of 15 BACs is indicated as a short vertical bar. All markers and transcripts are positioned according to the information provided by UCSC and NCBI. The horizontal axis (top) shows the number of FISH signals detected with each BAC probe. The number of signals was truncated at 10, because it was difficult to enumerate them above that level. Lines connect the measurements made for each cell line. The maximally amplified region was defined between BACs RP11-95J9 and 708L7 in AMO1 cells or between BAC RP11-25I9 and 708L7 in MOLP-2 cells, suggesting that shortest region of overlap (SRO) locates between BAC RP11-25I9 and 708L7. (Right) The nine known genes within the region of interest (between BAC RP11-25I9 and 708L7) that showed any expressions by reverse transcription–PCR (RT–PCR) analysis are identified (also see Table 1). Representative images of mRNA expression levels of known genes except *BTG4* (see Table 1), detected by RT–PCR in MM cell lines with (AMO1 and MOLP-2) and without (KMM1 and KM-5) 11q23 amplification, are shown. (**c**) Western-blot analysis of POU2AF1 protein in eight MM cell lines, using anti-POU2AF1 antibody. Anti-β-actin antibody was used for reprobing to show equivalent loading and transfer of proteins. The copy number of *POU2AF1* is given under the lane for each cell line. AMO1 and MOLP-2 cells, with amplified *POU2AF1*, showed overexpression of POU2AF1 at the levels of both mRNA and protein. (**d**) Copy-number ratios of *POU2AF1* in 23 primary cases of MM determined by real-time quantitative PCR. The abundance of the *POU2AF1* gene in genomic DNA of each sample was normalized by the corresponding endogenous control *GAPDH* value, and recorded as copy-number ratio. DNAs from five independent healthy volunteers were used as normal controls, and relative copy-number ratio of normal control DNA after normalization was 1.0 ± 0.28 (the mean $\pm$ s.d.). We used AMO1 and KMS-21BM cell line having amplification of *POU2AF1* as positive controls. Dotted line, the mean + 2 s.d. (1.56) indicating cutoff ratio for amplification.

significant. Among the nine known genes we tested, only *POU2AF1* matched that criterion in both AMO1 and MOLP-2 cell lines. Expression level of *POU2AF1* mRNA was not correlated with those with its interacting functional partners, Oct-1 and Oct-2 transcription factors (Supplementary Figure S2). Expression levels of POU2AF1 protein were consistent with their changes in copy number as well (Figure 2c).

Copy-number gain of POU2AF1 *in primary MM samples*

DNA copy number of *POU2AF1* in primary cases of MM was determined by a real-time quantitative PCR using genomic DNA obtained from 23 patients (Figure 2d). As the normal control of diploid copy number, we first determined the copy-number ratio of *POU2AF1* normalized with *GAPDH* in DNA derived from five independent healthy volunteers. We used

Table 1 Relative expression levels of positional candidate target genes for 11q23 gain/amplification in MM cell lines

Known gene[a]	*Amplified cells*		*Non-amplified cells*		*Fold increase (/reference value)*[b]	
	AMO1	*MOLP-2*	*KMM1*	*KM-5*	*AMO1*	*MOLP-2*
RDX	0.89	0.79	0.88	1.33		
FDX1	1.22	1.36	0.79	1.23		
ARHGAP20	0.48	1.71	1.39	2.93		
POU2AF1	0.54	0.60	0.06	0.18	**4.50**	**5.00**
BTG4	NT[c]	NT[c]	NT[c]	NT[c]		
LAYN	NT[c]	NT[c]	0.55	NT[c]		
SNF1LK2	0.75	0.32	0.60	0.51		
PPP2R1B	0.71	0.93	1.05	0.17		
ALG9	0.57	0.44	0.26	0.33	1.90	1.50

Abbreviations: *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; MM, multiple myeloma; SRO, smallest region of overlap. [a]Known genes were selected from the SRO of 11q23 amplicon in MM cell lines (Figure 2b). [b]The expression level of each gene in each cell line was divided by the average value of that in KMM1 and KM-5 cell lines (reference value), which have normal copy numbers of 11q, after normalization with *GAPDH*, and recorded as a fold increase in relative expression level. Fold increases in relative expression levels >2.0 were considered significant and are shown in bold type. The single gene that was significantly overexpressed as a consequence of amplification at 11q23 is highlighted with gray background. [c]NT indicates that expression level is below the lower limit of quantification.

AMO1 and KMS-21BM cell line having amplification of *POU2AF1* as positive controls. To separate cases with or without copy-number gain, the mean + 2 s.d. of copy-number ratio of *POU2AF1* in normal controls was used as the cutoff ratio (Inoue *et al*., 2004). The copy-number gain of *POU2AF1* was detected in 3 of 23 cases (13.0%), indicating that the *POU2AF1* amplification is not the artifact acquired during the establishment of cell lines, although a small number of cases were available for this analysis and the *POU2AF1* amplification was infrequently detected. However, this frequency of the *POU2AF1* amplification in MM is not so low compared with other gene amplification events: for example, *MYCN* amplification, one of the most critical genetic events determining prognosis in neuroblastoma, is known to be observed in less than 20% in this disease (Moreau *et al*., 2006).

Influence of POU2AF1 expression on proliferation of MM cells

Earlier studies showed that proliferation of POU2AF1$^{-/-}$ cells is diminished in response to surface IgM crosslinking, and those cells are unable to form germinal centers (Kim *et al*., 1996; Schubart *et al*., 1996). To investigate whether POU2AF1 can also influence proliferation of MM cells, we examined the effect of a decrease or increase in POU2AF1 expression in cell-growth assays. First, we introduced a *POU2AF1*-specific small-interfering RNA (siRNA) into AMO1 and KMS-21BM cells, which express POU2AF1 highly and moderately, respectively, and KMS-12PE and KMS-5 cells, which lack POU2AF1 amplification/overexpression (Figures 2b and c, and Table 1). Based on our experiments evaluating transfection efficiency using various concentrations of siRNA in MM cells, we used 200 nM of siRNA to obtain efficient transfection and knockdown effect (Supplementary Figure S3). Expression of POU2AF1 protein was knocked down 48 h after treatment with two different *POU2AF1*-siRNAs, compared with a nonspecific control siRNA, in MM cell lines (Figure 3a). Downregulation of POU2AF1 expression in AMO1 and KMS-21BM cells by siRNA decreased their growth (Figure 3b). *POU2AF1*-B-siRNA, which was more effective than the 'A' version in knocking down expression of POU2AF1 in AMO1 cells, also showed higher cell growth-inhibiting activity in that cell line. On the other hand, transfection of *POU2AF1*-siRNA showed almost no effect on growth of KMS-12PE and KMS-5 cells compared with a nonspecific control siRNA, suggesting that the growth inhibitory effect of *POU2AF1*-siRNAs observed in MM cells with amplification/overexpression of *POU2AF1* was caused by neither off-target effect nor simple nonspecific toxicity of siRNA used in this study.

Next, we established a cell line stably expressing exogenous POU2AF1 using KMS-11, which had shown low POU2AF1 expression. Between the two isoforms of POU2AF1, we chose p34 for transfection experiments because (a) the p34 isoform exhibited higher transactivating activity as a co-activator on octamer element-containing promoters than p35 and (b) p34 is derived from the p35 isoform through post-transcriptional modification (Yu *et al*., 2001). We determined expression of the Myc-tagged p34 isoform of POU2AF1 in selected clones by western blotting and by fluorescence immunocytochemistry (FIC), using anti-Myc epitope antibody (Figure 3c). Exogenously expressed POU2AF1 was observed mainly in nuclei, consistent with previous data (Yu *et al*., 2001). Cells stably expressing exogenous POU2AF1 showed increased growth compared with empty vector- (mock-) transfected cells (Figure 3d).

Regulation of TNFRSF17 *expression by POU2AF1*

An earlier report showed that expression of some non-Ig genes and/or proteins was affected in *POU2AF1*-deficient mice (Teitell, 2003). The candidate transcriptional targets for POU2AF1 included genes encoding BAFFR, TNFRSF17, Bcl-2, cyclin D3 and osteopontin (Samardzic *et al*., 2002; Brunner *et al*., 2003; Kim *et al*., 2003; Lins *et al*., 2003), proteins that might be

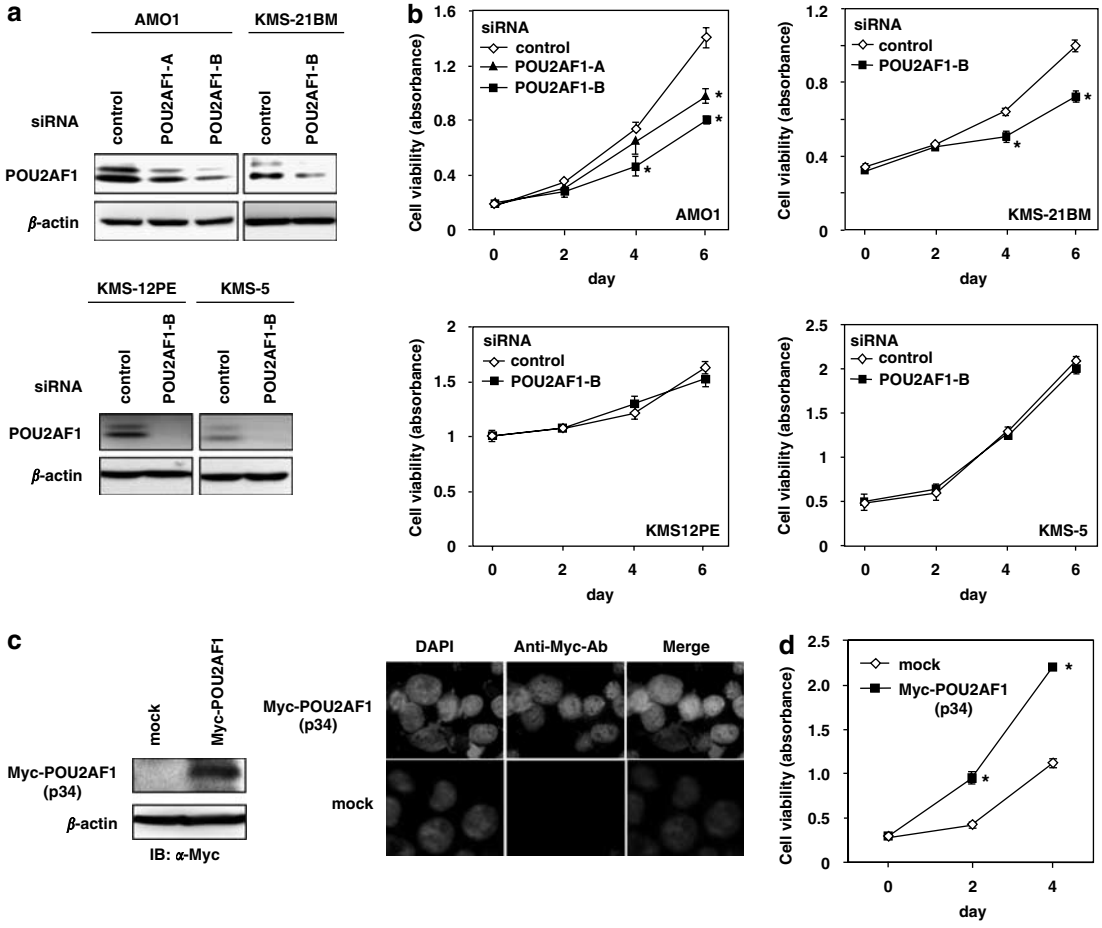

Figure 3 Effect of down- and upregulation of POU2AF1 expression on growth of multiple myeloma (MM) cells. (**a**) AMO1 and KMS-21BM cell lines, which have amplification/overexpression of *POU2AF1*, and KMS-12PE and KMS-5 cell lines, which lack amplification/overexpression of *POU2AF1*, were transfected with 200 nM nonspecific control small-interfering RNA (siRNA) or siRNA targeting POU2AF1 (*POU2AF1*-A and/or -B siRNA). A western blot of total cell lysates isolated 48 h after transfection was probed for POU2AF1, and reprobed for β-actin to show equivalent loading and transfer of proteins. (**b**) The relative number of living cells (cell viability) determined by colorimetric water-soluble tetrazolium salt (WST) assay was decreased by downregulation of POU2AF1 expression in *POU2AF1* siRNA-treated AMO1 and KMS-21BM cells, but not in KMS-12PE and KMS-5 cells, compared to nonspecific (control) siRNA-treated counterparts. $^*P<0.05$ with unpaired Student's *t*-test. (**c**) Myc-tagged POU2AF1 protein in stably transfected cells. (Left) Western-blot analysis of Myc-tagged POU2AF1 protein in stably transfected KMS-11 cells, which had shown low endogenous expression of POU2AF1. These cells were transfected with an expression construct containing Myc-tagged POU2AF1 isoform p34 (pCMV-Tag3-POU2AF1) or empty vector (pCMV-Tag3-mock) as a control, and selected by G418 for 3 weeks to establish stable lines. Anti-Myc antibody detected stably transfected Myc-tagged POU2AF1. (Right) Nuclear expression of stably transfected Myc-tagged POU2AF1 isoform p34, detected by fluorescence immunocytochemistry in KMS-11 transfectants, but not in mock vector-transfected control KMS-11 cells, using anti-Myc antibody and Texas red-conjugated anti-mouse antibody. Nuclei were stained with DAPI. (**d**) Increase in the relative number of living cells (cell viability) in KMS-11 cells stably expressing POU2AF1 compared with mock-transfected cells, determined by WST assay. $^*P<0.05$ with unpaired Student's *t*-test.

considered to participate in aspects of neoplastic development or progression such as proliferation, survival, cell cycling, adhesion or migration (Ladanyi *et al*., 1992; Claudio *et al*., 2002; Hideshima *et al*., 2004; Novak *et al*., 2004; Standal *et al*., 2004; Heckman *et al*., 2006). To investigate whether, as a transcriptional co-activator, POU2AF1 regulates expression of those genes in MM cells, we examined the mRNA level of each of them in MM cells treated with *POU2AF1*-siRNA or in a MM cell line stably expressing POU2AF1, by real-time quantitative RT–PCR. Among the five candidates, only *TNFRSF17* mRNA was decreased in AMO1 and KMS-21BM cells treated with *POU2AF*1 siRNA compared with control cells treated with nonspecific siRNA (Figure 4a and data not shown), and elevated in cells stably expressing POU2AF1 compared with cells transfected with wild vector alone (mock, Figure 4a). Relatively small increase and decrease in *TNFRSF17* mRNA expression through POU2AF1 overexpression and knockdown in MM cells (Figure 4a) suggest that *TNFRSF17* transcription may also be regulated or modified by other regulatory transcription factors or cofactors in this cell lineage.

Using a transcription element search software package, RecGroupScan (http://www.mgs.bionet.nsc.ru/mgs/programs/yura/RecGropScanStart.html), we found

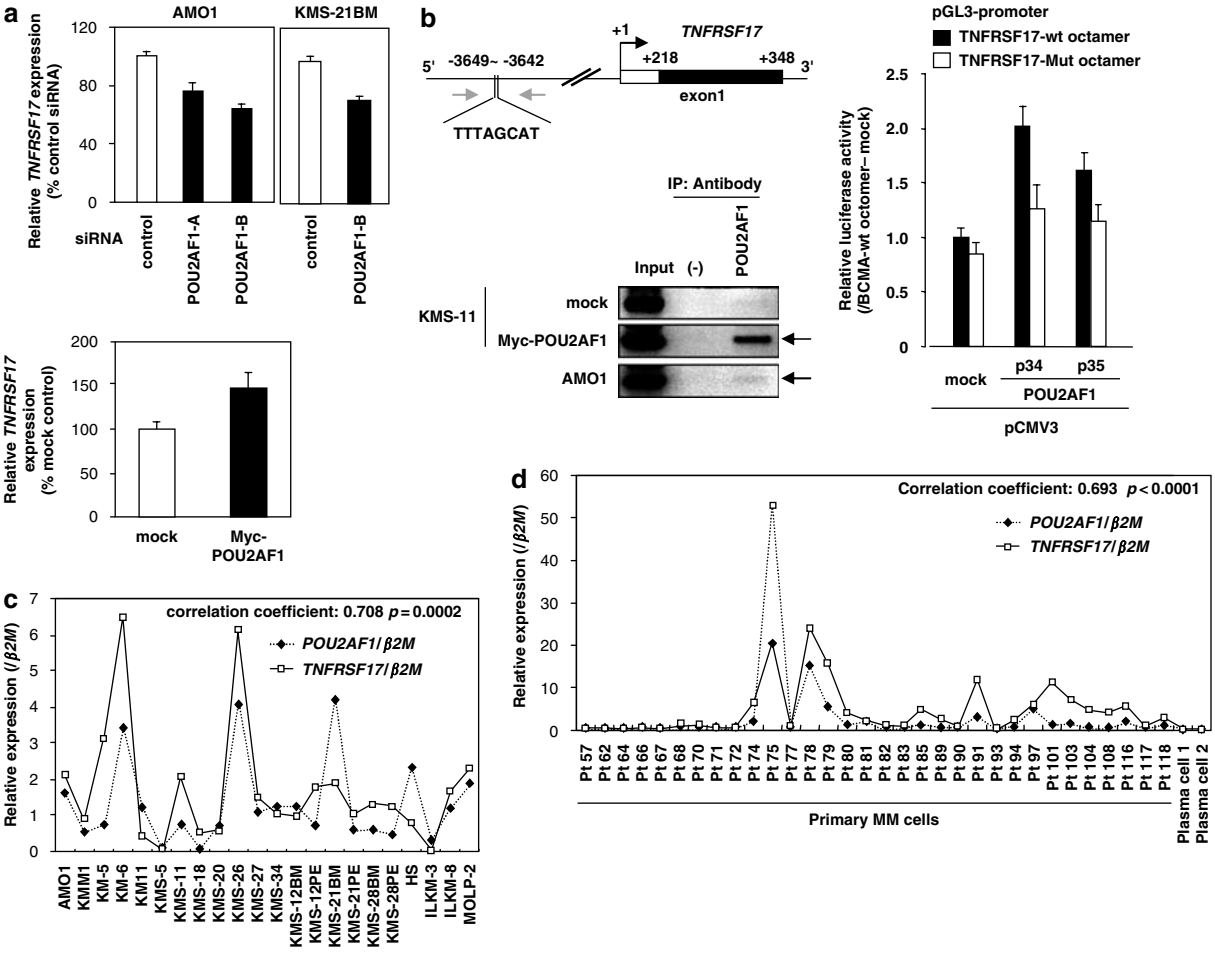

Figure 4 (**a**) Effect of down- and upregulation of POU2AF1 expression on TNFRSF17 mRNA expression. (Upper) AMO1 and KMS-21BM cell lines were transfected with 200 nM nonspecific control small-interfering RNA (siRNA) or siRNA targeting *POU2AF1* (*POU2AF1*-A and/or -B siRNA), and expression of *TNFRSF17* mRNA was examined by quantitative reverse transcription–PCR (RT–PCR) 48 h after transfection. For normalization, values were adjusted to β-actin levels, and % relative expression levels were calculated based on designating the nonspecific levels as 100. (Lower) Expression of *TNFRSF17* mRNA in a transfected KMS-11 cell line stably expressing POU2AF1 and in mock vector-transfected KMS-11 cells, determined as described above. (**b**) *TNFRSF17* as a direct transcriptional target for POU2AF1. (Left upper) Diagrammatic representation of the 5′ region of *TNFRSF17* and the octamer site identified within it, based on the results of a transcription element search using RecGroupScan software (http://www.mgs.bionet.nsc.ru/mgs/programs/yura/RecGropScanStart.html). Positions relative to the transcriptional start site (+1) of the *TNFRSF17* gene are shown. White and black boxes indicate 5′ untranslated and coding regions of exon 1, respectively. Arrows denote primers used for ChIP assay. (Left lower) Binding of POU2AF1 to the endogenous *TNFRSF17 cis*-regulatory region in multiple myeloma (MM) cells. Chromatin immunoprecipitations from AMO1 cells and from transfected KMS-11 cells stably expressing POU2AF1 were performed as described in Materilas and methods. PCR products were resolved on 3% agarose by electrophoresis. DNA from lysates before immunoprecipitation was used as positive input control. The primer set amplified the region containing an octamer site (arrows). The experiment was repeated twice. (Right) Reporter assays of wild type and a point mutant of the octamer. Equal amounts of reporters containing either wild or mutant octamer-site sequences were introduced into cells with *Renilla* luciferase vector, along with expression vectors for POU2AF1 isoforms p34 or p35, or empty vector (mock) alone. Firefly luciferase activities in samples were normalized to *Renilla* luciferase activities in the same specimens, and relative luciferase activity was calculated based on considering the value in cells with wild-type octamer and mock expression vector as 1. The data presented are the mean$\pm$s.d. of three separate experiments, each performed in triplicate. (**c**) Correlation between expression levels of *POU2AF1* and *TNFRSF17* mRNAs in MM cell lines. The mRNA level of each gene was measured by quantitative RT–PCR, as described in Materials and methods. Expression levels were normalized to that of *β2-microglobulin* (*β2M*). (**d**) Correlation between expression of *POU2AF1* and *TNFRSF17* mRNAs in 32 primary MM specimens as well as in normal plasma cells from two individuals. Expression levels were normalized to that of *β2M*.

an octameric motif upstream of the transcription start site of *TNFRSF17* (Figure 4b). To determine whether POU2AF1 directly regulates the transcription of this gene in MM cells, we performed chromatin immunoprecipitation (ChIP) assays to examine whether a transcriptional complex containing POU2AF1 could occupy this motif *in vivo*, by using AMO1 cells and KMS-11 cells stably expressing POU2AF1. In both cell populations, the POU2AF1 complex bound to the region containing the specific octamer upstream of

TNFRSF17 (Figure 4b). We then tested the transactivating activity of POU2AF1 on a 307-bp fragment encompassing that specific octameric sequence, in KMS-11 cells. As shown in Figure 4b, p35 and p34 isoforms of POU2AF1 showed clear enhancement of the *TNFRSF17*-wt octameric site; this effect was significantly inhibited by changing the octamer's sequence from wild-type (TTTAGCAT) to mutant (TTCCGCAT).

Correlation of POU2AF1 *and* TNFRSF17 *expression in MM cell lines and primary MM cells*

To examine the relevance of *TNFRSF17* regulation by POU2AF1 *in vivo*, we determined mRNA levels of *POU2AF1* and *TNFRSF17* by quantitative real-time RT–PCR in 22 MM cell lines and in MM cells purified from bone marrow aspirates of 32 patients with MM. Significant correlation between mRNA levels of the two genes was observed in the cell lines (correlation coefficient: 0.708, $P=0.0002$, the Pearson's correlation test; Figure 4c) and in the primary MM cells (correlation coefficient: 0.693, $P<0.0001$, the Pearson's correlation test; Figure 4d). Notably, case Pt75, Pt78 and Pt97, which showed *POU2AF1* amplification in genomic PCR (Figure 2d), also showed relatively higher mRNA expression of this gene and *TNFRSF17*. mRNA expression levels of *POU2AF1* and *TNFRSF17* in normal plasma cells isolated from two individuals were lower than those in primary MM cells (0.023 and 0.003 versus 3.107 ± 9.580 in *POU2AF1*, and <0.001 and <0.001 versus 4.620 ± 5.989 in *TNFRSF17*; mean $\pm$ s.d.), even though only two normal cases were available for analysis. These observations provided evidence that (a) *POU2AF1* is overexpressed through either its amplification or other mechanisms in MM cells and (b) *TNFRSF17* resides selectively in a transcriptional pathway with *POU2AF1* within terminally differentiated B cells, even within a neoplastic lineage.

Promotion of MM cell growth by TNFRSF17, downstream of POU2AF1

To test whether TNFRSF17 affects cell growth as transcriptional target for POU2AF1, we treated MM cells overexpressing POU2AF1 with a *TNFRSF17*-specific siRNA. Since no anti-TNFRSF17 antibody suitable for western blotting was available, we first determined levels of mRNA expression between *POU2AF1* and *TNFRSF17* by quantitative real-time RT–PCR analysis, 48 h after transfection with their respective siRNAs. When we treated the cells with *POU2AF1* siRNA, expression of both mRNAs was decreased, but only *TNFRSF17* mRNA expression was inhibited by *TNFRSF17* siRNA (Figures 5a and b). This result confirmed *TNFRSF17* as a downstream target of POU2AF1. After treatment with *TNFRSF17* siRNA, as with *POU2AF1* siRNA, growth of AMO1 and KMS21BM cells was significantly inhibited compared to growth of the same cells treated with a nonspecific (control) siRNA (Figure 5c). Combined with the results described above, these data suggest that overexpressed POU2AF1 accelerates growth of MM cells by activating expression of *TNFRSF17*.

Discussion

Abnormalities of chromosome 11, especially 11q, which can involve translocations and/or gains or losses of DNA, are common events in MM (Tricot *et al*., 1995). Gains of 11q in plasma cells have been reported in 20–45% of MM patients, ranging from trisomies to single chromosomal bands (Cigudosa *et al*., 1998; Gutierrez *et al*., 2001). Aberration of 11q13 is probably due to cyclin D1, which is overexpressed as a result of translocation or an amplification mechanism (Sonoki *et al*., 1998; Hoechtlen-Vollmar *et al*., 2000). The targets in other affected regions of 11q, such as 11q23 and 11q25 (Gonzalez *et al*., 2004; Carrasco *et al*., 2006), remain unknown. Using in-house array-CGH, we detected high-level amplification at 11q23 in two MM cell lines, AMO1 and MOLP-2. After analysing all nine known genes located within the SRO of this amplicon, we found that POU2AF1, a B-cell-specific transcriptional co-activator, was consistently overexpressed in MM cell lines that exhibited increased copy numbers in that region. Functional analyses in combination with one of its possible transcriptional targets, *TNFRSF17*, suggested that *POU2AF1* is a likely target of amplification in MM and, when overexpressed by any mechanism, contributes to the pathogenesis of the disease by transactivating a specific downstream transcriptional target, *TNFRSF17*.

POU2AF1 functions as an essential and nonredundant co-activator by binding Oct-1 and Oct-2 transcription factors. It is required for transcriptional activity at the octameric DNA element ATGCAAAT and similar regulatory sequences (Cepek *et al*., 1996; Gstaiger *et al*., 1996). Since expression of POU2AF1 is largely restricted to B lymphocytes, the functions of this protein in normal B-cell development have been extensively studied. In mice, although disruption of the POU2AF1 locus resulted in failure to form germinal centers and reduced levels of secondary Ig isotypes, B cells from POU2AF1$^{-/-}$ mice were similar to normal resting B cells (Nielsen *et al*., 1996; Schubart *et al*., 2001). Additionally, mice in which the lymphoid compartment was replaced with cells lacking both Oct-2 and POU2AF1, B-cell development and Ig expression were mostly unchanged, although the population of mature peripheral B cells was reduced (Schubart *et al*., 2001). These studies suggested that POU2AF1 and Oct factors are not as important for expression of immunoglobulins as much as for determination of cell fate and maintenance of the B-cell population.

Little information is available regarding the role of POU2AF1 in malignant transformation and proliferation in B-cell lineages. Overexpression of POU2AF1 has been observed in germinal center-derived lymphomas (Greiner *et al*., 2000). Recently, Largo *et al*. (2006) reported that POU2AF1 locates within frequently

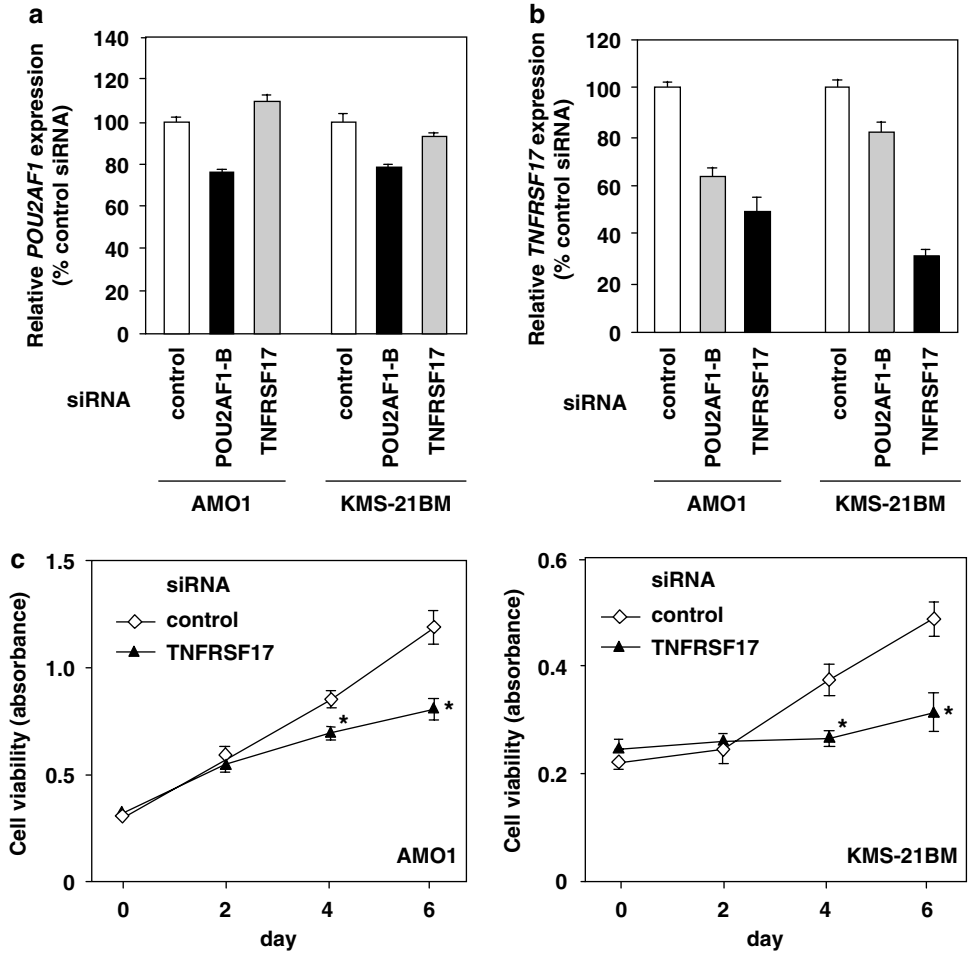

Figure 5 Effect of downregulated TNFRSF17 on growth of multiple myeloma (MM) cells. (**a**) and (**b**) Expression of *POU2AF1* mRNA (**a**) or *TNFRSF17* mRNA (**b**) in AMO1 and KMS-21BM cells transfected with 200 nM of either nonspecific control small-interfering RNA (siRNA), an siRNA targeting *POU2AF1* or *TNFRSF17* siRNA. Quantitative reverse transcription–PCR (RT–PCR) analyses were performed 48 h after transfection. For normalization, values were adjusted to β-actin levels, and % relative expression levels were calculated based on setting the nonspecific control siRNA levels as 100. (**c**) Decrease in relative number of living cells (cell viability), determined by colorimetric water-soluble tetrazolium salt (WST) assay, after downregulation of *TNFRSF17* expression in *POU2AF1* siRNA-treated AMO1 and KMS-21BM cells compared to control siRNA treated cells. $^*P<0.05$ with unpaired Student's *t*-test.

gained region and is overexpressed regardless of copy-number status in MM cells compared with universal human RNA. Heckman *et al*. (2006) reported that Oct factors and POU2AF1 mediate cell survival in t(14;18) lymphoma cells by directly activating the antiapoptotic gene *Bcl*-2, and they may therefore play a role in malignant transformation. However, they evaluated only the number of apoptotic cells, not total cell growth or the numbers of proliferative cells, after knockdown of those factors. Since studies with *POU2AF1*-deficient B cells have indicated that POU2AF1 targets genes are responsible for B-cell proliferation and signaling (Kim *et al*., 2003) and that expression of the antiapoptotic factor Bcl-2 in POU2AF1$^{-/-}$ mice is able to rescue early B lymphopoiesis but not B-cell responsiveness (Brunner *et al*., 2003), a primary role of POU2AF1 and Oct elements might be to act as cell-growth and/or survival factors in normal and malignant development of B-cell lineages. Our experiments using stable transfectants expressing POU2AF1 and siRNA-induced knockdown of POU2AF1 in cell lines highly expressing the gene successfully demonstrated the growth-promoting effect of overexpressed POU2AF1 in human MM cells. Expression of bcl-2 was not correlated with amplification or expression levels of POU2AF1 in the MM cell lines we examined (data not shown), suggesting that this protein may have different transcriptional targets and might show different effects among various malignancies.

Since POU2AF1 is a specific co-activator at octameric promoters, its growth-promoting effect on MM cells may be due to its ability to activate transcription of certain target gene(s). Despite the fact that a large number of genes specifically expressed in B cells contain octamer motifs in their regulatory regions, only a small number of POU2AF1-dependent genes have been described, among them, BAFFR, TNFRSF17, bcl-2, cyclin D3 and osteopontin (Samardzic *et al*., 2002;

Brunner *et al*., 2003; Kim *et al*., 2003; Lins *et al*., 2003). We have now confirmed that of this group of genes, only *TNFRSF17* serves as a transcriptional target for POU2AF1 in MM cells. Therefore, POU2AF1 may activate genes differently in MM cells than in cells belonging to normal B-cell lineages, or t(14;18) lymphoma cells. Indeed, others have shown that in MM cells, TNFRSF17 is expressed with its ligand BAFF, a B-cell-activating factor, which modulates the proliferative capacity of cytokine-stimulated MM cells through its ability to promote survival (Schneider *et al*., 1999; Novak *et al*., 2004). Although two other BAFF receptors, TACI and BAFF-R, are also expressed in MM cells (Novak *et al*., 2004), TNFRSF17 may function as an important receptor of BAFF to transduce signals for cell survival and proliferation, given that TNFRSF17 is a member of the TNFR superfamily lacking a 'death domain' and given the fact that its overexpression in HEK293 cells activates nuclear factor-κB, p38 and c-Jun N-terminal kinase (Hatzoglou *et al*., 2000). Using gene-specific siRNAs and ChIP assays, we demonstrated that TNFRSF17, when transcriptionally induced by POU2AF1, contributes to growth of MM cells. In our ongoing study (C Zhao and J Inazawa, unpublished data), we also confirmed that a list of genes possibly transactivated by POU2AF1, which was compiled by our preliminary expression-array analysis using *POU2AF1*-B-siRNA-transfected U266 myeloma cell line, in a comparison with nonspecific siRNA-transfected counterparts, included *POU2AF1*, but did not include other four genes.

Of the two isoforms of POU2AF1, p34 and p35, p34 is the predominant isoform expressed in MM cells (Figure 2c). Others have noted that p34 had higher transcription activity than p35 (Yu *et al*., 2001), and our experiments using reporter constructs containing the Oct sequence upstream of *TNFRSF17* (Figure 4b) confirmed it. Therefore, we introduced isoform p34 into the KMS-11 cell line to establish POU2AF1-stable transfectants that exhibited greater growth than their parent MM cells, suggesting that at least the p34 isoform of POU2AF1 could exhibit oncogenic activity during myelomagenesis. However, since p35 also affected expression of TNFRSF17 (Figure 4b), even though weakly, it is possible that both isoforms can contribute to growth of MM cells.

Recent reports suggested that augmented expression of POU2AF1 does not occur at the transcriptional level (Husson *et al*., 2002; Robetorye *et al*., 2002; Heckman *et al*., 2006), and that post-translational modification of POU2AF1 may contribute to its stability and activity in t(14;18) lymphoma cells (Tanaka and Herr, 1990; Zwilling *et al*., 1997). However, t(14;18) lymphoma cells treated with specific siRNA have shown decreases in both *POU2AF1* mRNA and its protein product (Heckman *et al*., 2006). In this study, we demonstrated that (a) both *POU2AF1* mRNA and protein were upregulated in MM cells where amplification had occurred at 11q23.1 and (b) the level of *POU2AF1* mRNA was correlated with that of one of its transcriptional targets, *TNFRSF17*. These data suggest that POU2AF1 expression, at least in MM cells, is regulated at least partially at the transcriptional level. The mechanism(s) responsible for augmented POU2AF1 expression in various malignancies, including MM, need to be elucidated.

In summary, the study presented here has demonstrated that *POU2AF1* is a target for amplification at 11q23 in MM. POU2AF1 mediates growth of MM cells, at least in part, by activating one of its transcriptional targets, TNFRSF17. As the POU2AF1–TNFRSF17 pathway appears to be important for myelomagenesis, it represents a potentially suitable target for development of novel reagents for treatment of MM, a hematological malignancy that is not curable at present.

Materials and methods

MM cell lines and primary MM specimens

We examined 28 human MM cell lines established from primary samples, (AMO1, KMM1, KM-1, KM-4, KM-5, KM-6, KM-7, KM-11, KMS-5, KMS-11, KMS-18, KMS-20, KMS-26, KMS-27, KMS-34, KMS-12BM, KMS-12PE, KMS-21BM, KMS-21PE, KMS-28BM, KMS-28PE, HS, ILKM-10, ILKM-12, ILKM-13, MOLP-2, MOLP-6 and OPM-2) and one Epstein–Barr virus-transformed normal lymphocyte cell line. All lines were maintained in RPMI-1640 supplemented with 10% fetal bovine serum. Genomic DNA was available from all cell lines; RNA and chromosomal spreads were available from 22 of them.

Primary MM specimens were obtained from 32 patients who were undergoing treatment in Nagoya City University Hospital. At the time of diagnosis, 1–2 ml of bone marrow aspirate was obtained from each patient after written informed consent according to guidelines approved by the Institutional Ethical Committee. MM cells were purified from the populations of mononuclear cells in marrow aspirates by positive selection, using anti-CD138 antibody-coated beads and an automatic magnetic cell sorting system (Miltenyi Biotec, Auburn, CA, USA) as described elsewhere (Miura *et al*., 2003). Normal plasma cells corrected from patients with non-neoplastic diseases were enriched using the same method described as above. RNA was isolated from all specimens, whereas genomic DNA was available from 23 of 32 MM patients.

Array-CGH analysis

In-house MCG Cancer Array-800 contains 800 BAC/PAC clones carrying genes or sequence-tagged-site markers of potential importance in carcinogenesis (http://www.cghtmd.jp/cghdatabase/index.html; Inazawa *et al*., 2004). Hybridizations were carried out as described elsewhere (Sonoda *et al*., 2004). The hybridized slides were scanned with a GenePix 4000B (Axon Instruments, Foster City, CA, USA). Acquired images were analysed with GenePix Pro 6.0 imaging software (Axon Instruments). Fluorescence ratios were normalized so that the mean of the middle third of $\log 2$ ratios across the array was zero. The thresholds for copy-number gain and loss were set at $\log 2$ ratios of 0.4 and -0.4, respectively (Takada *et al*., 2005), and we defined $\log 2$ ratios >2.0 and <-2.0 as high-level amplifications and homozygous deletions, respectively.

Fluorescence in situ *hybridization*

Metaphase chromosomes were prepared from normal male lymphocytes and from each MM cell line. fluorescence *in situ*

hybridization analyses were performed, as described elsewhere (Inoue *et al*., 2004), using BACs located around the region of interest as probes. The copy-number and molecular organization of the region of interest were assessed according to the hybridization patterns observed on both metaphase and interphase chromosomes.

Semiquantitative RT–PCR and quantitative real-time genomic and RT–PCR

Semiquantitative expression analyses were performed by RT–PCR with all transcripts that emerged within the 11q23 amplicon. Single-stranded cDNA was generated from total RNA. PCR products were electrophoresed in 3% agarose gels, and bands were quantified using LAS-3000 (Fujifilm, Tokyo, Japan) and Multi Gauge software (Fujifilm). Quantitative real-time genomic PCR and RT–PCR experiments were performed using an ABI Prism 7900 sequence-detection system (Applied Biosystems, Foster City, CA, USA) as described previously (Inoue *et al*., 2004; Takada *et al*., 2005). Genomic copy number in each primary sample was normalized by dividing it by the corresponding *GAPDH* value, which is located at 12p13 and rarely involved in MM (Inoue *et al*., 2004), and recorded as copy-number ratios. Each assay was performed in duplicate. All primer sequences for amplifying genes chosen for this study are available on request.

Western blotting

Anti-POU2AF1, Myc tag and β-actin antibodies were purchased, respectively, from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Cell Signaling Technology (Beverly, MA, USA) and Sigma (St Louis, MO, USA). Cells were lysed in RIPA buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 1% sodium deoxycholate, 0.1% SDS and 1% Triton X-100, pH 7.4) with a protease-inhibitor cocktail (Roche, Tokyo, Japan). Protein samples were separated by SDS–polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride membranes. Proteins were detected after incubation with appropriate primary antibodies and peroxidase-conjugated secondary antibodies, followed by development with an enhanced electrochemiluminescence system (Amersham, Tokyo, Japan).

FIC

MM cells were centrifuged using Shandon Cytospin (Thermo, Pittsburgh, PA, USA) onto slide glasses, fixed with 4% formaldehyde, permialized with 0.2% Triton X-100 and incubated with polyclonal anti-Myc antibody (Cell Signaling Technology) overnight. Signals were visualized using Alexa 488 goat-anti rabbit antibody (Molecular Probes, Eugene, OR, USA), and the labeled fluorescence was examined by fluorescence microscopy.

Transfection with siRNA and cell-growth assays

POU2AF1-specific siRNA was purchased from Japan Bio Services (Saitama, Japan; *POU2AF1*-A siRNA: CACCUUA CACCGAGUAUGU, *POU2AF1*-B siRNA: GGUUCUGU GUCUGCAGU). *TNFRSF17*-specific siRNA (*TNFRSF17* siRNA M-011217-00) and a nonspecific control siRNA (D-001206-11-20) were purchased from Dharmacon (Lafayette, CO, USA). For each experiment, 2×10^6 MM cells in logarithmic growth phase were transfected with either 200 nM *POU2AF1*, *TNFRSF17* or control siRNA using Nucleofector (Amaxa, Koeln, Germany) following the manufacturer's protocol. High transfection efficiency of siRNA experiment using this system had been confirmed by our preliminary study using nonspecific control siRNA labeled with fluorescent dye (siGLO RISC-Free siRNA, Dharmacon) in each cell line (85 ± 9 and 75 ± 6% in AMO1 and KMS-21BM, respectively; the mean $\pm$ s.d.; data not shown). Transfection efficiency in each experiment was monitored by fluorescein-labeled pmaxGFP analysis. Knockdown efficiency was determined 48 h after transfection by RT–PCR. For measurements of MM cell growth, 1×10^4 cells were seeded into 96-well plates after transfection and the numbers of viable cells were assessed by a colorimetric water-soluble tetrazolium salt (WST) assay (Cell counting kit-8; Dojindo Laboratories, Kumamoto, Japan). Experiments were repeated two times, and each series was performed in triplicate.

Construction of expression plasmids and establishment of stable transfectants

Plasmids expressing a Myc-tagged p34 or p35 isoform of POU2AF1 (pCMV-Tag3-p34-POU2AF1 or pCMV-Tag3-p35-POU2AF1, respectively) were obtained by cloning the full coding sequence of each isoform into the pCMV-Tag3 vector (Stratagene, La Jolla, CA, USA) in-frame, along with the Myc-epitope. For establishment of sub-lines that stably expressed the Myc-tagged p34 isoform of POU2AF1, KMS-11 cells were transfected with pCMV-3-p34-POU2AF1 and pCMV-3B empty vector using Nucleofector (Amaxa). Transfected cells were selected with G418 (50 μg/ml) for 3 weeks, and independent clones were established by limiting dilution. Individual clones were analysed for ectopic POU2AF1 expression by western blotting and FIC.

ChIP

ChIP assays were performed as described previously (Sonoda *et al*., 2004). Chromatin was immunoprecipitated with anti-POU2AF1 antibody (Santa Cruz Biotechnology), after which PCR was performed with 1/100 of the immunoprecipitated DNA; 1/100 of the solution before adding antibody was amplified as an internal control for the amount of DNA.

Promoter reporter assay

Since an octamer motif at position 5 and/or 6 is critical for the transactivation activities of POU2AF1 (Cepek *et al*., 1996; Gstaiger *et al*., 1996), we designed a mutant octamer site containing two cytosines at those positions. PCR-amplified upstream regions of *TNFRSF17* containing wild-type (TTTAGCAT) or mutant (TTCCGCAT) octamer sites were cloned into pGL3-promoter vector (Promega, Madison, WI, USA), and the resulting constructs were designated pGL3-TNFRSF17-wt octamer or pGL3-TNFRSF17-Mut octamer, respectively. Each construct was introduced into KMS-11 cells along with pCMV-Tag3-POU2AF1 isoform p34 or p35 and phRL-TK internal control vector (Promega), using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Firefly luciferase and *Renilla* luciferase activities were each measured 48 h after transfection by means of the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity, normalized with *Renilla* luciferase activity, was divided by the value found in cells transfected with pGL3-TNFRSF17-wt octamer or control vector pCMV-Tag3 (mock) and the difference was recorded as a fold increase in relative luciferase activity.

Statistical analyses

The unpaired Student's *t*-test was used to compare the significance of differences in cell growth patterns under different treatments. To assess the significance of the correlation, the Pearson's correlation test was used between mRNA expression levels of *POU2AF1* and *TNFRSF17* after each was normalized; $P < 0.05$ was considered significant in each case.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).